Molecular characterization of freshwater microalgae and nutritional exploration to enhance their lipid yield

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Abstract

Biomass and lipid yield under nutrient depleted and supplemented conditions of N and Mg in two freshwater isolates, *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 from Meghalaya were investigated for biodiesel production. Both the strains, *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 are oleaginous in nature having lipid content of 40.2 ± 1.4 and $37.3 \pm 2.6\%$ DCW, respectively. The significant increase (92.8%) in lipid content was found in N-depleted condition while an increase (46.65%) in biomass yield was observed under Mg-supplemented condition in *Chlorella* sp. NC-MKM. Studying the interactive effects of nutrient depletion and supplementation, combination of N-depleted and Mg-supplemented condition was selected for further investigation to check enhanced lipid yield in *Chlorella* sp. NC-MKM. The results showed a significant increase in biomass yield, lipid yield and lipid content (30, 66.8, and 28.66%, respectively). Under this condition, accumulation of neutral lipid was also enhanced (47.17% M2 gated cells) compared to control (21.37% M2 gated cells). Further, FAMES revealed that the relative percentage of saturated and mono-unsaturated fatty acids increased (66.16%) in *Chlorella* sp. NC-MKM compared to control that improves biodiesel properties.

Keywords Microalgae · Lipid yield · FAMES · Fuel properties · Biodiesel

Introduction

The demand for biofuel production is increasing day by day being a renewable, sustainable and ecofriendly energy source. The rapid depletion of fossil fuels and increase in global warming are main issues behind enhanced biofuel research across the world (Hepbasli 2008). Biodiesel is mainly derived from biomass of plants and microalgae (Sheehan et al. 2011). Microalgae have been identified as a better source of lipids that can be trans-esterified to produce biodiesel (Malcata 2011; Parmar et al. 2011). Sheehan et al. (2011) reported that amount of lipid produced per acre by microalgae greatly exceeds compared to agricultural oleaginous crops. Oil yield of microalgal feedstock

is 38,000–50,800 liters/hectare/year (L/ha/year) while from soyabean it is 450 L/ha/year (Darzins et al. 2010). Hence, microalgae seem to be the best source of lipid or feedstock for biodiesel production. Various marine and freshwater strains of microalgae have been isolated and characterized across the world but the identification of a suitable feedstock is yet to be done.

Numerous research studies imply that under stress conditions, microalgae change their biosynthetic pathways to increase production of lipid (Fan et al. 2014; Gwak et al. 2014). Nutrient depletion/supplementation, high salinity, high/low temperature, heavy metals, etc., are stress factors which increases lipid content. However, they are unable to increase biomass as well as lipid productivity altogether. For instance, under nitrogen (N) stress conditions, biosynthesis of total lipid and neutral lipid increases up to 50.32 and 34.29% dry cell weight (DCW), respectively, in *Chlorella pyrenoidosa* (Fan et al. 2014). The triacylglycerol production under N stress condition increases from 22 to 36% DCW in *Neochloris oleoabundans* as reported by Rismani-Yazdi et al. (2012). The deficiency of nutrients such as phosphorus (P), potassium (K), iron (Fe), magnesium (Mg) and calcium (Ca) also influence the accumulation of a large amount of

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neutral lipid (Deng et al. 2011). However, under P-depleted and thiosulphate-supplemented condition, total lipid content increased up to 30% DCW in *Scenedesmus obliquus* (Mandal and Mallik 2009). Xin et al. (2010) have found 53% increase in lipid content in *Scenedesmus* sp. under the P-limited condition. The deficiency of Ca and K in SE or BG11 medium noticeably increases lipid content in *Chlorella vulgaris*. Limitation of Mg in HSM or BG11 medium, led to higher cellular lipid level (Deng et al. 2011). Gorain et al. (2013) have reported that the lipid content was increased 40% DCW compared to 11.9% in control for *C. vulgaris* and 37% DCW compared to 11.3% control for *S. obliquus* under Ca depletion condition.

Likewise, the supplementation of nutrient elements also influences the accumulation of lipid but few studies have been reported yet. Gorain et al. (2013) have reported that the total lipid content was increased up to 27 and 26% DCW for *C. vulgaris* and *S. obliquus*, respectively, at 2× concentration (100 mg/L) of Mg on 15th day of incubation. In *Dunaliella tertiolecta*, the lipid productivity increased threefold (47.4 mg/L/Day against 13.3 mg/L/Day) after ten times of N supplementation (Mata et al. 2013). However, *Picochlorum* sp. was observed with 0.6% decrease in lipid content than control in 100% N supplementation (El-Kassas 2013). However, reports on supplementation of other nutrients with their effect on increase in lipid productivity are lacking.

Biodiesel fuel properties mainly depend on the fatty acid methyl ester (FAME) as the key fuel properties such as cetane number (CN), saponification value (SV), iodine value (IV), kinematic viscosity (ν), cold filter plugging point (CFPP) and higher heating value (HHV) derived from it (Knothe 2005; Islam et al. 2013). The CN is a primary indicator of fuel quality as it indicates ignition delay time, which is the time between injection and ignition. The higher the CN, shorter the ignition delay time, and vice versa (Gopinath et al. 2009). IV determines oxidative stability of biodiesel (Islam et al. 2013). Furthermore, biodiesel must have a suitable kinematic viscosity (ν) to make sure that a sufficient fuel supply reaches injectors at different operating temperatures (Ramírez-Verduzco et al. 2012). As reported that the higher percentage of saturated fatty acids (SFA) and mono-unsaturated fatty acids (MUFA) leads to better oxidative stability and energy yield from biodiesel while poly unsaturated fatty acids (PUFA) maintains the cold flow properties of biodiesel (Islam et al. 2013).

Each nutrient element has their impact on lipid and biomass induction. N is an important constituent of protein and enzyme, depletion of it induces high lipid induction but decreases the biomass productivity. Mg is the central atom element of the photosynthetic apparatus, cofactors of enzymes. Hence, it plays a very important role in biomass yield but only few studies reported its impact on lipid yield.

In the present study, effect of the nutrient elements such as N and Mg in both (depleted and supplemented) conditions will be studied to understand its regulatory role in improving the lipid productivity in microalgae.

Materials and methods

Algae isolation and culture conditions

Microalgal samples were collected from Nohkalikai falls (Cherrapunji, East Khasi Hills District, Meghalaya, India). Samples were grown in BG11 medium (Rippka and Herdman 1992) with a photoperiod of 16/8 light: dark, light intensity of 2500 lx at a temperature of 25 °C. Isolation and purification were performed by serial dilution and streak plate technique. Identification of pure culture was done on the basis of microscopic observation of culture under compound microscope (Olympus) at 100×.

Genomic DNA extraction, PCR amplification of 18S rRNA gene and sequencing

The genomic DNA was extracted from two freshwater algal isolates using plant genomic DNA extraction kit (HiMedia) and stored at −20 °C until used for PCR amplification. The oligonucleotide primers 18S forward-Euk328f 5'-ACC TGG TTG ATC CTG CCA G-3' (Moon-van der Staay et al. 2000) and 18S reverse-Chlo02r 5'-CTT CGA GCC CCC AAC TTT C-3' (Zhu et al. 2005) were used for PCR amplification of partial 18S rRNA gene. The PCR amplification was performed in 2720 thermocycler (Applied Biosystems). The PCR products were examined by electrophoretic separation on 1.2% agarose gels and sequencing was done by Eurofins Genomics India Pvt. Ltd.

Phylogenetic analysis

The 18S rRNA gene sequences of the isolates obtained were searched for homology in NCBI database using BLAST. The partial 18S sequences were assembled in DNASTAR software and aligned using CLUSTAL-W. The phylogenetic analysis was performed by MEGA6 software (Tamura et al. 2013). The phylogenetic tree was constructed using neighbor joining method. The bootstrap values were calculated from 1000 replicates and shown as percentages.

Growth and nutrient depletion/supplementation studies

To study the effect of nutrient depletion and supplementation on lipid content, biomass and lipid yield of both the isolates, two nutrient elements nitrogen and magnesium

were selected. The compounds used for N and Mg stress are NaNO_3 and MgSO_4 , respectively. The content of NaNO_3 in medium had been depleted from 17.6 mM (control) to 8.8 mM, 4.4 mM and 2.2 mM and supplemented to 19.8, 22, 26.4 and 35.2 mM while MgSO_4 depleted from 304 (Control) to 152, 76 and 38 μM and supplemented to 342, 380, 456 and 608 μM to observe their effect on selected microalgal isolates.

The experiment was carried out in Erlenmeyer flasks of 250 mL, containing 150 mL BG11 for 18 days (Rippka and Herdman 1992). The growth of identified algal isolates was monitored by spectrophotometric (SmartSpec™ Plus, Bio-Rad, USA) analysis. Optical density was observed at 750 nm in an interval of one day till 18 days. Biomass yield was calculated based on gravimetric method and expressed in mg/L. All measurements were carried out in triplicate. All chemicals were analytical grade and purchased from Sigma-Aldrich (USA) and HiMedia (India).

Lipid extraction and estimation

Lipid extraction was performed using method as described by (Gwak et al. 2014). The dried cell pellet was grinded and mixed with solution of chloroform: methanol: water (2:2:1.8, v/v) and vigorously agitated. Phase separation was performed upon centrifugation at 1000g for 5 min and lower organic phase was collected and evaporated the solvent. The dry weights were measured as total lipid. Lipid content and lipid yield were expressed as % DCW and mg/L.

Flow cytometric analysis of intracellular lipid

The accumulation of intracellular lipid was assessed by flow cytometer (Becton Dickinson Instruments, Franklin Lakes, NJ, USA) in association with fluorescent dye Nile red. The fluorescence signal of Nile red-stained cells was obtained using laser filter (FL2-H) with an excitation of 488 nm and an emission of 586/42 nm for neutral lipid. Microalgal cells were stained with 3.0×10^{-3} μM Nile red dye in 1 mL of volume reaction, incubated at 37 °C for 15 min.

The unstained cells were used as an instrument control. Cell size and cell granularity were compared with two scattering signals (FSC and SSC) by flow cytometry. Percentage (%) grandparents of stained cell population were expressed as fluorescence arbitrary units.

Fluorescent microscopy

Microalgal cells were stained with Nile red dye (10 μg /mL). After staining, the slides were prepared using 10% glycerin (v/v) and observed under fluorescence microscope (Leica Microsystems CMS GmbH, Germany). The fluorescent signals were captured using an excitation wavelength

of 470 nm, and emission was collected between 560 and 600 nm (Huang et al. 2009; Chen et al. 2009).

FAMES preparation and analysis by GC–MS

Transesterification was performed using slightly modified method described by Li et al. (2013). The lyophilized microalgal powder was blended with 1.5 mL of $\text{NaOH-CH}_3\text{OH}$ and the mixture was saponified in a water bath at 75 °C for 10 min. Then 2 mL of boron trifluoride–methanol (1:2, v/v) was added and again kept in a water bath at 75 °C for 10 min. The phase separation was carried out using 0.9% NaCl solution and 2 mL of hexane. Finally, the upper layer fatty acid was collected and subjected to GC–MS analysis. Samples were analyzed using a gas chromatograph (PerkinElmer Clarus 680 GC/600C MS, USA) equipped with a mass-selective detector, an autosampler, split/splitless injector, an injection module and GC–MS software (Turbomass Ver5.4.2). Elite-5MS Capillary Column was used. Helium gas was used as carrier gas at a flow rate of 1.00 mL/min; the split ratio was 50:1 and injection volume was 1.0 μL . The flame ionization detector (FID) was used at temperature 60–280 °C. Fatty acid methyl esters were identified by auto scan and direct comparison of their mass spectra pattern. The retention index was determined with the NIST library mass spectral database (S/W Turbomass NIST 2008).

Calculation of fuel properties from FAMES

Various important biodiesel properties (such as CN, SV, IV, CFPP, ν , ρ , HHV) were estimated directly from the FAMES. CNs of FAMES were calculated using the following equation (Knothe 2006):

$$\text{CN} = 46.3 + \frac{5.458}{\text{SV}} - (0.225 \times \text{IV}),$$

where CN, SV and IV are the cetane number, saponification value and iodine value respectively. The SV in mg KOH/g and IV in g I_2 /100 g of fat were estimated by the following equations (Krisnangkura 1986):

$$\text{SV} = \sum_i \left[\frac{(560 \times N_i)}{\text{MW}_i} \right],$$

$$\text{IV} = \sum_i \left[\frac{(254 \times D_i \times N_i)}{\text{MW}_i} \right],$$

where N_i is the percentage of i th FAME; D_i is the number of double bonds of the i th FAME and MW_i is the molecular weight of i th FAME.

The degree of unsaturation (DU) was calculated based on the mass fraction of MUFA and PUFA (Ramos et al. 2009):

$$DU = \sum \text{MUFA} + (2 \times \text{PUFA}).$$

The long chain saturation factor (LCSF) and the CFPP in °C were estimated based on (Ramos et al. 2009):

$$\text{LCSF} = (0.1 \times C16 : 0) + (0.5 \times C18 : 0) + (1 \times C20 : 0) + (2 \times C24 : 0),$$

$$\text{CFPP} = (3.1417 \times \text{LCSF}) - 16.477.$$

The ν , ρ and HHV of each FAMEs were calculated and summation of all FAME-derived fuel properties provided the final ν , ρ and HHV based on (Ramírez-Verduzco et al. 2012):

$$\ln(\nu_i) = -12.503 + 2.946 \times \ln(\text{MW}_i) - 0.178 \times D_i,$$

$$\rho_i = 0.8463 + \frac{4.9}{\text{MW}_i} + (0.0118 \times D_i),$$

$$\text{HHV}_i = 46.19 - \frac{1794}{\text{MW}_i} - (0.21 \times D_i),$$

where ν_i is the kinematic viscosity at 40 °C in mm^2/s ; ρ_i is the density at 20 °C in g/cm^3 ; and HHV_i is the higher heating value in MJ/kg of i th FAME.

Analysis of data

The data were analyzed using GraphPad Prism 5 and graphs were plotted using Origin Pro. 8.0. All the measurements were carried out in triplicates.

Results and discussion

Characterization of microalgal strains

In the present study, two indigenous microalgal isolates were identified and the effect of depletion and supplementation of N and Mg examined to enhance lipid yield (Fig. 1a, b). Preliminary morphological identification of algal isolates on the basis of microscopic observation showed that the isolates (NC-MKM and NC-M2) belonged to the genus *Chlorella* (Fig. 2a) and *Scenedesmus* (Fig. 2b), respectively. Genus *Chlorella* is unicellular green algae belong to the family *Chlorellaceae* and class *Trebouxiophyceae*. The cells are solitary, spherical and globular in shape. The cells are without flagella, having cupped-shaped chloroplast and single pyrenoid surrounded with thin wall (Tale et al. 2014). The morphological characteristic of isolate NC-M2 resembles to genus *Scenedesmus* which belongs to the family *Scenedesmaceae*

and class *Chlorophyceae*. The cells are non-motile, elongated in shape with pointed ends, contains a chloroplast with pyrenoid (Tale et al. 2014). Metzger and Largeau (2005) reported that morphology of algae could vary in relation to age and culture condition within each chemical race and for the same strain. To avoid any variance arising out of morphological heterogeneity, phylogenetic analysis of partial 18S rRNA gene

sequences of these two isolates was carried out to supplement morphological observations. The phylogenetic analysis illustrated that *Chlorella* sp. NC-MKM isolate with accession number KY048406 is closely related to *Chlorella* sp. AK-2013 with accession number KC790434 (98% similarity), whereas *Scenedesmus acutus* NC-M2 isolate with accession number KY129792 is closely related to *Scenedesmus acutus* strain NL accession number KF746929 (99% similarity) (Fig. 3a, b). The molecular data provides more trustworthy results to the phylogeny of algae (Soylu and Gonulol 2012).

Biomass and lipid yield as well as lipid content in *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 under batch culture mode were estimated in BG11 medium of standard composition. The biomass, lipid yield and lipid content of *Chlorella* sp. NC-MKM were found 251 ± 7.07 , 101.4 ± 7.7 mg/L and $40.3 \pm 1.4\%$ DCW while *Scenedesmus acutus* NC-M2 was 281 ± 12.5 , 104.8 ± 4.5 mg/L and $37.3 \pm 2.6\%$ DCW, respectively, under normal condition (Fig. 4a–c). The growth of both microalgae studied under nutrient depleted and supplemented condition with respect to given concentration present in standard medium (Supplementary Figs. S1–S3). In the two isolates, *Scenedesmus acutus* NC-M2 was observed with higher growth rate compared to *Chlorella* sp. NC-MKM (Figs. S1 and S2). However, both the strains possessed high lipid content; hence have great potential to be used as feedstock for biodiesel production.

Effect of N depletion and supplementation

Nutrient depletion is an efficient trigger to elevate lipid accumulation in microalgae and it has been reported by many other researchers (Hu et al. 2008; Rismani-Yazdi et al. 2012; Fan et al. 2014). In our study, decreasing the concentration of N in BG11 medium also showed a positive effect on lipid content, and at 2.2 mM concentration lipid content was increased up to $77.7 \pm 1.3\%$ DCW (92.8% rise) for *Chlorella* sp. NC-MKM and $67.36 \pm 2.8\%$ DCW (80.42% rise) for *Scenedesmus acutus* NC-M2 (Fig. 4a). But significant cut in biomass proportionately leads to a decrease in lipid yield in both *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 (72 ± 2.8 , 56 ± 5.6 and 47.5 ± 3.5 , 32.1 ± 0.14 mg/L respectively) (Fig. 4b, c). Increase in lipid content may be attributed to the carbohydrate intermediate metabolites

Fig. 1 Flow chart of work design **a** *Chlorella* sp. NC-MKM and **b** *Scenedesmus acutus* NC-M2. LC, BY and LY stand for lipid content, biomass and lipid yield, respectively. + symbol stands for increase amount while – symbol for decrease amount of respective parameters

Fig. 2 Microscopic images of algal isolates under light microscope. **a** *Chlorella* sp. NC-MKM, **b** *Scenedesmus acutus* NC-M2

Fig. 3 A dendrogram depicting the position of *Chlorella* sp. strain NC-MKM and *Scenedesmus acutus* strain NC-M2 within the radiation of members of the family *Chlorellaceae* and *Scenedesmaceae*. The strains used in this study are boxed. The scale bar shows five nucleotide exchanges per thousand nucleotides

generated during acetate assimilation which are utilized to synthesize triacylglycerols (TAGs) by the Kennedy pathway (Turpin 1991; Deng et al. 2011). Since, the overall production of lipid yield reduced significantly (Fig. 4c). This could be due to the decrease in biomass yield under N-depleted condition. However, several reports suggested that the N limitation induces significant increase in lipid content along with decrease in biomass and lipid productivity in *C. pyrenoidosa*, *N. oleoabundans* and *Chlorella sorokiniana* (Hu et al. 2008; Rismani-Yazdi et al. 2012; Fan et al. 2014).

Whereas supplementation of N showed a positive effect on biomass yield, and at 26.6 mM concentration biomass yield was elevated up to 318.5 ± 9.04 mg/L against 251 ± 7.07 mg/L (26.69% rise) for *Chlorella* sp. NC-MKM and for *Scenedesmus acutus* NC-M2 at 35.2 mM concentration, 14.3% of increase recorded (Fig. 4e). The increase in biomass production was associated with decrease in lipid content and lipid yield for both *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 ($23.4 \pm 0.8\%$ DCW, 63.1 ± 0.7 mg/L and $21.45 \pm 2.05\%$ DCW, 61.8 ± 4.8 mg/L respectively) as noticed (Fig. 4d, f). High dose of N in culture medium induced large biomass yield due to significant increase in protein and nucleic acid biosynthesis which further promotes cell growth but decreases the total lipid content (Deng et al. 2011; Belotti et al. 2013). Li et al. (2015) also found similar patterns of increasing biomass and decreasing lipid content under the N-supplemented condition.

Effect of Mg depletion and supplementation

The evaluation of the effect of Mg depletion and supplementation on biomass yield, lipid yield and lipid accumulation potential of *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 were also conducted (Fig. 5a–f). The

standard concentration of Mg in BG11 medium is 304 μ M. Depletion of Mg concentration illustrated upbeat effect on the lipid accumulation ability of both the algal isolates. At 38 μ M concentration of Mg, an increase in lipid content was recorded for *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 (36 and 28% respectively) (Fig. 5a). Biomass and lipid yield for both the isolates showed a reducing pattern (Fig. 5b, c). The depletion of Mg in culture medium is expected to hinder cell division, cessation of chlorophyll synthesis, and hence, biomass yields (Finkle and Appleman 1953). Gorain et al. (2013) have reported similar effect of Mg starvation in *C. vulgaris* and *S. obliquus*. The slight increase in lipid content could be attributed to the stress of nutrient depletion which may promote lipid biosynthesis by carbon redistribution and starch conversion (Zhang et al. 2017).

However, increasing concentration of Mg showed positive effect on biomass yield for *Chlorella* sp. NC-MKM, whereas no significant difference was noticed for *Scenedesmus acutus* NC-M2. Profound rise (46.65%) in biomass yield, i.e., 368.1 ± 13 mg/L against 251 ± 7.07 mg/L was estimated under supplementation (608 μ M) of Mg for *Chlorella* sp. NC-MKM (Fig. 5e). Lipid content shows a decreasing trend while no significant difference was observed on lipid yield for *Chlorella* sp. NC-MKM (Fig. 5a, c). Higher concentration of Mg induced high biomass yield as Mg plays a vital role in photosynthesis, respiration, and adenosine triphosphate production (Li et al. 2014). Increased in biomass with supplementation of Mg in growth medium was also reported by previous workers (Finkle and Appleman 1953; Sydney et al. 2010). Ulloa et al. (2012) reported that the supplementation of excess Mg to the culture medium decreases the lipid content and lipid yield in *Tetraselmis suecica*, whereas Sydney et al. (2010) reported that the Mg supplementation to the culture medium increases both biomass and lipid yield in *D. tertiolecta*, *C. vulgaris*, *Spirulina platensis* and *Botryococcus braunii*.

Effect of interaction of N depletion and Mg supplementation

Since economical feasibility of biodiesel production depends on lipid yield, therefore, further experiments were carried out to get better lipid yield. For this, *Chlorella* sp. NC-MKM was selected to examine the interactive effect of N-depleted (4.4 mM) and Mg-supplemented (608 μ M) condition on biomass and lipid yield. Previous experiments showed that the N-depleted (4.4 mM) condition leads to significant increase in lipid content (50%) and Mg-supplemented (608 μ M) condition leads to a higher increase in biomass yield (46.65%) compared to control in *Chlorella* sp. NC-MKM. The results stated that the biomass and lipid yield as well as total lipid content significantly increase, i.e., 30,

Fig. 4 Estimation of lipid content, biomass and lipid yield of *Chlorella* sp. NC-MKM under nutrient depleted (a–c) and supplemented (d–f) conditions. Data represent the mean values of triplicates and

error bars show the standard deviation. Asterisk indicates significant difference between control and treatments according to one-way ANOVA (Tukey's test) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

66.81 and 28.66% (326.23 ± 5.6 mg/L, 169.15 ± 8.13 mg/L/Day and $51.85 \pm 1.9\%$ DCW against 251 ± 7.07 mg/L, 101.4 ± 7.7 mg/L and $40.3 \pm 1.4\%$ DCW, respectively)

compared to control under combined condition of N depletion (4.4 mM) and Mg supplementation (608 μ M) in *Chlorella* sp. NC-MKM (Fig. 6a–c). Under this condition, all the

Fig. 5 Estimation of lipid content, biomass and lipid yield of *Scenedesmus acutus* NC-M2 under nutrient-depleted (a–c) and -supplemented (d–f) conditions. Data represent the mean values of triplicates

and error bars show the standard deviation. Asterisk indicates significant difference between control and treatments according to one-way ANOVA (Tukey's test) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

three parameters biomass, lipid yield and total lipid content were significantly increased (Fig. 6a–c). The possible reason for this could be that in early stage, high Mg and sufficient N concentration in culture medium may supported a profound increase in biomass production, whereas N depletion in later stages lead to higher lipid accumulation, hence, increased lipid yield.

Neutral lipid analysis under N-depleted and Mg-supplemented condition

The biodiesel production is mainly depends on neutral lipid (Triacylglycerol) production, as biodiesel (i.e., FAMES) derives from esterification of neutral lipid (TAG). Hence, *Chlorella* sp. NC-MKM under combination of N-depleted and Mg-supplemented condition was selected for further study of neutral lipid production. Figure 7a–f illustrates the effect of N depleted and Mg supplemented condition on the neutral lipid accumulation for *Chlorella* sp. NC-MKM. The FSC versus SSC area plot demonstrated uniformly scattered cells population containing neutral lipid after Nile red staining (Fig. 7a, d). The flow cytogram of *Chlorella* sp.

NC-MKM under N-depleted and Mg-supplemented condition showed high fluorescence intensity in cells stained with Nile red compared to control condition (Fig. 7b, e). The accumulation of neutral lipid was estimated based on the percentage (%) gated cells parameter under N-depleted and Mg-supplemented condition compared to control cells. It was observed that under N-depleted and Mg-supplemented condition, the neutral lipid content of *Chlorella* sp. NC-MKM increased (47.17% M2-gated cells) compared to control (21.37% M2-gated cells). Fluorescent images of Nile red-stained cells under N-depleted and Mg-supplemented condition showed a large number of cells with yellow-gold color (neutral lipid droplets) compared to control (Fig. 7c, f). These data are fortified by the fact that nitrogen-depleted condition results rise in neutral lipid accumulation in microalgae, cyanobacteria and diatoms (Hsieh and Wu 2009; Yeh and Chang 2011; PraveenKumar et al. 2012; Sun et al. 2014). Satpati and Pal (2015) have illustrated a flow cytometric approach with Nile red staining method to detect neutral lipid synthesis of *Chlorella ellipsoidea* and *Chlorococcum infusionum* under nitrate-depleted conditions and reported similar results. The fluorescent images of *Chlorella*

Fig. 6 Estimation of total lipid content, lipid and biomass yield (a–c, respectively) of *Chlorella* sp. NC-MKM under combination of N-depleted and Mg-supplemented conditions [–N + Mg]. Data represent the mean values of triplicates and error bars show the standard deviation. Asterisk indicates significant difference between control and treatments according one-way ANOVA (Tukey's test) (* $P < 0.05$). C stands for control (standard) condition of nutrient elements

Fig. 7 Flow cytometric analysis of Nile red-stained cells of *Chlorella* sp. NC-MKM. **a** and **d** Forward scatter (FSC) versus side scatter (SSC) two-dimensional (2D) dot plots of stained cells under standard condition and combined condition of N-depleted and Mg-supplemented, respectively. **b** and **e** Flow cytograms of stained cells under standard condition and combined condition of N-depleted and Mg-supplemented, respectively. **c** and **f** Neutral lipid bodies of Nile red-stained cells under standard condition and combined condition of N-depleted and Mg-supplemented, respectively

sp. NC-MKM stained with Nile red showed more number of cells with yellow-gold color under N-depleted and Mg-supplemented conditions compared to control (Fig. 7c, f). The yellow-gold color of fluorescence of Nile red-stained cells corresponds to the neutral lipid droplets while the red-dish color is suggestive of auto fluorescence of pigments and polar lipids (Satpati and Pal 2015). Hence, the results of fluorescent images are in great agreement with data obtained by flow cytometric approach.

FAMES

The fatty acid composition directly influences the fuel properties of algae-based biodiesel. Therefore, it is imperative to investigate the fatty acid composition whether it could be used for high quality biodiesel production. In the

present work, standard and N-depleted (2.2 mM) conditions were selected for FAMES analysis of both the microalgal isolates and combination of N-depleted (4.4 mM) and Mg-supplemented (608 µM) conditions were selected for further analysis of FAMES of *Chlorella* sp. NC-MKM, as these conditions showed highest increase in total lipid content and lipid yield, respectively (FAME peaks; Fig. S4). Our results illustrated that palmitic acid ($37.20 \pm 2.1\%$) and linoleic acid ($45.75 \pm 0.9\%$) were predominant in *Chlorella* sp. NC-MKM, whereas palmitic acid ($27.3 \pm 1.5\%$), hexadecatetraenoic acid ($15.1 \pm 0.6\%$) and eicosatrienoic acid ($45.7 \pm 1.3\%$) in *Scenedesmus acutus* NC-M2 under standard condition (Table 1). Under N-depleted (2.2 mM) condition, the synthesis of oleic acid (MUFA) was recorded highest (i.e., $42.48 \pm 1.02\%$) than any other fatty acid in *Chlorella* sp. NC-MKM. *Scenedesmus acutus* NC-M2 was

Table 1 FAME profile of *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 investigated by GC–MS

Particulars		Relative percentage of fatty acids				
Fatty acid methyl ester (FAME)						
Chemical formula	Common name	CC ^a	CT ^b	SC ^c	ST ^d	NC-2 ^e
C10:0	Pelargonic acid	–	–	–	–	2.14 ± 0.02
C12:3		6.04 ± 0.03	4.51 ± 0.1	–	–	–
C14:0	Myristic acid	–	–	–	–	22.73 ± 1.2
C16:0	Palmitic acid	37.20 ± 2.1	37.57 ± 2.2	27.3 ± 1.5	40 ± 3	9.41 ± 0.2
C16:2		–	–	–	–	21.5 ± 2
C16:4		4.97 ± 0.04	–	15.1 ± 0.6	8.7 ± 0.2	–
C18:0	Stearic acid	6.01 ± 0.03	4.89 ± 0.01	2.4 ± 0.03	–	–
C18:1	Oleic acid	–	42.48 ± 1.02	–	4.6 ± 0.1	37.42 ± 1.2
C18:2	Linoleic acid	45.75 ± 0.9	10.54 ± 0.2	9.5 ± 0.1	–	–
C18:4	Stearidonic acid	–	–	–	–	6.75 ± 0.3
C20:3		–	–	45.7 ± 1.3	46.7 ± 1.2	–
%SFA		43.24 ± 2	42.09 ± 2	29.7 ± 1.5	40 ± 3	34.27 ± 1.4
%USFA		56.76 ± 0.89	57.91 ± 0.81	70.3 ± 0.77	60.0 ± 0.9	65.73 ± 0.5
%MUFA		–	47.37 ± 1.01	–	4.6 ± 0.1	37.42 ± 1.2
%PUFA		56.76 ± 0.89	15.43 ± 0.2	70.3 ± 0.77	55.4 ± 1	28.31 ± 0.7
% SFA + MUFA		43.24 ± 2	84.57 ± 1.08	29.7 ± 1.5	44.6 ± 3.1	71.69 ± 1.1

Data represent the mean values of triplicates and ± shows the standard deviation

^a*Chlorella* sp. NC-MKM under control (C) condition

^b*Chlorella* sp. NC-MKM under nitrogen-depleted (2.2 mM) condition

^c*Scenedesmus acutus* NC-M2 under control (C) condition

^d*Scenedesmus acutus* NC-M2 under nitrogen-depleted (2.2 mM) condition

^e*Chlorella* sp. NC-MKM under combination of N-depleted (4.4 mM) and Mg-supplemented (608 µM) condition

found with a rise in the production of palmitic acid (SFA), i.e., 40 ± 3% against 27.3 ± 1.5% under N-depleted (2.2 mM) condition. Hence, under N-depleted (2.2 mM) condition, the relative percentage of SFA and MUFA content increase in both *Chlorella* sp. NC-MKM (95.58% rise) and *Scenedesmus acutus* NC-M2 (50.16% rise) compared to control (i.e., 84.57 ± 1.08% against 43.24 ± 2% and 44.6 ± 3.1% against 29.7 ± 1.5%, respectively) (Table 1). While under combined N-depleted (4.4 mM) and Mg-supplemented (608 µM) conditions, *Chlorella* sp. NC-MKM was found with predominance of myristic acid (22.73 ± 1.2%), oleic acid (37.42 ± 1.2%) and hexadecadienoic acid (21.5 ± 2%). Hence, under this condition, the relative percentage of SFA and MUFA content increases up to 66.16% compared to control (71.69 ± 1.1% against 43.24 ± 2%) in *Chlorella* sp. NC-MKM (Table 1). The biodiesel produced from both the microalgae under control condition was predominated with unsaturated fatty acid (USFA) components; might be due to their adaptation in low temperature and low light intensity condition of Meghalaya, India (Table 1). It has been reported that microalgae increases the production of unsaturated fatty acid (USFA) under low temperature condition to maintain the fluidity of cellular membrane (Wagenen et al. 2012). The accumulations of SFA and MUFA under these conditions

are supported by the fact that nutrient starvation condition results in more biosynthesis SFA and MUFA (Gorain et al. 2013).

Biodiesel properties based on FAMES

The biodiesel fuel properties are directly related to their fatty acid compositions. The main biodiesel fuel properties such as CN, IV, SV, CFPP, LCSF, DU, ν , ρ , and HHV, derived from FAMES of *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 are shown in Table 2. CN value is related to ignition delay time and combustion quality of diesel. High CN ensures good cold start properties and reduces the formation of white smoke and NO_x emission (Ladommatos et al. 1996; Gorain et al. 2013). In this study, maximum CN values 57.08 and 49.20 were observed for *Chlorella* sp. NC-MKM under N-depleted (2.2 mM) condition and combination of N-depleted (4.4 mM) and Mg-supplemented (608 µM) conditions, respectively. Under both the stress conditions, *Chlorella* sp. NC-MKM was observed a significant increase in SFA and MUFA that improves biodiesel properties by rising CN value. Total unsaturation in biodiesel is measured by IV. The polymerization of glycerides and deposition of

Table 2 Biodiesel properties derived from the FAME profile of *Chlorella* sp.

Sample	SV (mg KOH g ⁻¹)	IV (g I ₂ 100 g ⁻¹ fat)	CN	LCSF	CFPP (°C)	DU	Density (ρ) (g cm ⁻³)	HHV (MJ kg ⁻¹)	Kinematic viscosity (ν) (mm ² s ⁻¹)
BS ^a	–	≤ 120	≥ 51	–	≤ 5/≤ -20	–	0.86–0.9	NA	3.5–5.0
BS ^b	–	NA	≥ 47	–	NA	–	NA	NA	1.9–6.0
CC ^c	202.34	120.52	46.20	6.72	4.64	113.5	0.88	38.95	3.38
CT ^d	199.26	73.8	57.08	5.83	1.84	76.19	0.86	39.26	3.89
SC ^e	190.7	182.8	33.79	3.9	–4.21	141	0.88	39.61	3.81
ST ^f	190.2	147	42	4	–3.9	114	0.87	39.55	3.93
NC-2 ^g	203	109	49.2	0	0	112.8	0.8799	39.59	3.08

NC-MKM and *Scenedesmus acutus* NC-M2. The values of biodiesel standards EN 14214 and ASTM D6751-02 were from Islam et al.

^aBiodiesel standards EN 14214

^bBiodiesel standards ASTM D6751-02

^c*Chlorella* sp. NC-MKM under control (C) condition

^d*Chlorella* sp. NC-MKM under nitrogen-depleted (2.2 mM) condition

^e*Scenedesmus acutus* NC-M2 under control (C) condition

^f*Scenedesmus acutus* NC-M2 under nitrogen-depleted (2.2 mM) condition

^g*Chlorella* sp. NC-MKM under combination of N-depleted (4.4 mM) and Mg-supplemented (608 μ M) conditions

lubricant in the engine is mainly caused by higher IV of the biodiesel (Francisco et al. 2010). As stated by European standards EN 14214, IV of biodiesel should be less than 120 g I₂/100 g of fat (Knothe 2005). In the present study, *Chlorella* sp. NC-MKM was observed with lower IV, i.e., 73.8 g I₂/100 g of fat under N-depleted (2.2 mM) condition and 109 g I₂/100 g of fat under combined N-depleted (4.4 mM) and Mg-supplemented (608 μ M) conditions. CFPP is the most important property for low temperature applications. This property is highly affected by the presence of SFA in biodiesel (Sharma et al. 2016). Both *Chlorella* sp. NC-MKM and *Scenedesmus acutus* NC-M2 examined in this study showed CFPP ranges from –4.21 to 4.64. According to European standards EN 14214, CFPP of biodiesel should be in the range of ≤ 5/≤ –20. *Chlorella* sp. NC-MKM under both the conditions N-depleted (2.2 mM) and combination of N-depleted (4.4 mM) and Mg-supplemented (608 μ M) conditions were observed with the prescribed viscosity range 3.38–3.08 mm²/s, respectively, therefore meeting both standards (Table 2). *Chlorella* sp. NC-MKM under both the conditions were found in the prescribed density range with 0.86–0.87 g/cm³, therefore meeting the standards for biodiesel. HHV is the indication of energy produced by the combustion of biodiesel. The FAME-derived HHVs of *Chlorella* sp. NC-MKM under both conditions were found in the range of 13.8–13.9% less than obtained from petroleum-derived diesel (46 MJ/kg) (Ramírez-Verduzco et al. 2012).

Conclusions

The *Chlorella* sp. NC-MKM under combination of N-depleted (4.4 mM) and Mg-supplemented (608 μ M) conditions showed significant 66.81% increase in lipid yield and enhanced neutral lipid content. The biodiesel fuel properties of this found in accordance with the ASTM D6751-02 and EN14214 standard for biodiesel fuel properties, thus further studies may provide clearer view for biodiesel production at large scale.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical approval The manuscript does not contain experiments on animals and human; hence ethical permission not required.

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